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Observation on So-called "Saliva of the Aphid" in Relation to the Virus Infection

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Summary

The experiments were carried out for examining the "saliva" which has been used generally in experimentation until now.

From the experiments, it is obvious that artificially collected aphid saliva is contaminated with bacteria due to the insertion of the stylets. Also, that the virus infection is inhibited by bacterial products. Therefore it is suggested that one needs to avoid the contamination of microorganisms when the so-called "saliva" is collected for experiments on the relation between the saliva of aphid and the virus infection.

It has been generally known that the solution on which the aphids were fed through the parafilm or thin rubber membrane included watery saliva, and that there are some reports done about solutions of aphid saliva. The authors have investigated the relation between aphid saliva and virus infection. However, it was very difficult to obtain the saliva in sufficient amounts for experiments. Moreover, the authors have some questions about the saliva collected through the membrane generally. The first, as feeding solution may contain only a very minute volume of saliva, it is doubtful whether we can obtain the true salivary components by the concentration or alcohol sedimentation of feeding solution. And the second, it has not been done under an aceptic conditions thus allowing contamination by microorganisms or microbial products in the feeding solution, so it becomes questionable that the inhibitory effect of the saliva on the virus infection known generally is due to the salivary material itself. We will present here the latter of these two questions.

Materials and Methods

(1) *Sterilization of parafilm membrane bag*

The bag made of a stretched thin parafilm membrane (Marathon Corporation, Wisconsin, U.S.A.) was inserted into the glass tube (30 mm in diameter) as

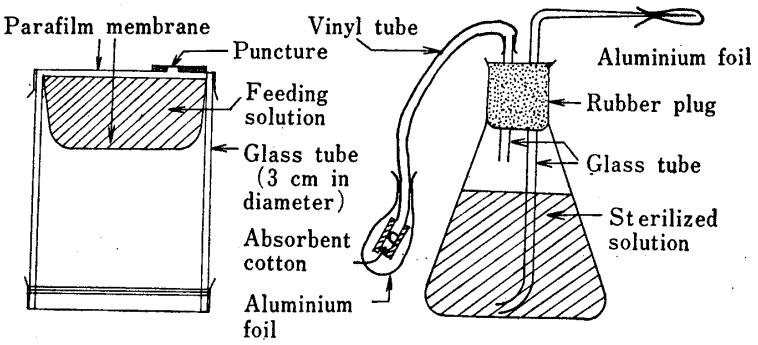

Fig. 1. Illustration of the experimental equipment

indicated in Fig. 1. This membrane bag was filled up with 80% ethylalcohol and then the upper part of the bag was sealed with another thin parafilm membrane. Next, the tube was shaken and the alcohol solution attached itself to the entire inner surface of the parafilm membrane. After 5 minutes, the upper membrane was punctured by a sterilized needle to remove the alcohol solution. Because a few drops of alcohol remained on the inner surface of the parafilm membrane, sterilized distilled water was injected for washing and then removed. Sterilization and washing were repeated three times. The inside of the bag was perfectly sterilized by this procedure.

(2) *Aphids*

Non-viruliferous green peach aphid (*Myzus persicae* Sulz.) of the apterous viviparae type which had been reared on Japanese radish (*Raphanus sativus* L.) were used for the experiments. After fasting for 3 hours, 300 aphids were placed in the glass tube for feeding on the solution through the membrane and the glass tube was covered with cheese cloth to prevent the escape of the aphids.

(3) *Partial purification of the virus*

An ordinary strain of bean yellow mosaic virus (BYMV-O) was used in this experiment. Crude virus preparation was obtained from BYMV-infected broad bean leaves (*Vicia faba* L. *var.* Wase soramame) using the method described by Wetter and Paul (1962). The virus was partially purified from the crude preparation by the centrifugation.

Broad bean leaves infected with BYMV was homogenized in 0.01 M phosphate buffer (pH 7.0) under low temperature. Fifty grams of leaves per 50 ml of the buffer solution were used. The homogenate was filtered through a gauze and then ascorbic acid and Na_2SO_3 were added to bring the final concentration to 0.2% respectively. The filtrate was centrifuged at 10,000×g for 30 minutes under a cold condition. After ultracentrifugation of the supernatant fluid at 78,000×g for 90 minutes, the sedimented pellet was resuspended in 10 ml of 0.01 M borate buffer (pH 7.0) and the suspension was centrifuged at 10,000×g for 10 minutes. Then

two cycles of centrifgation at 78,000×g and 10,000×g were repeated and thus partially purified virus was obtained.

Results

I. Microorganisms detected from fed solution

Whether or not microorganisms are introduced into the fed solution by stylet insertion was investigated.

Two ml of sterilized Husain and Kelman medium were injected into the sterilized parafilm membrane bag. Three hundred aphids were placed in the lower part of the glass tube and allowed to feed on the solution through the parafilm membrane for 0.5, 1, 10 and 24 hours. Then the aphids were removed from glass tube after a given period. The feeding solution of 0.5 ml was collected with a sterilized pipette and poured on Bouillon agar medium and then spread with a sterilized glass stick. After incubation for two days at 25°C, bacterial colonies which appeared on the medium were observed. Examination of fungi was done by using PDA (Potato Dextrose Agar) medium as cited above.

No fungi were detected but bacteria were detected from the solution on which the aphids fed (Table 1). Two types of bacterial colonies were observed from the solution fed for 30 minutes to 1 hour and only one type was observed after feeding of 10–24 hours. Although the name of this predominant bacterium is not known, its colony has a milky color, and the bacterium is gram-negative and rod-shaped (about 0.4 $\mu \times 1.2$ μ).

TABLE 1. *Microorganisms isolated from fed solution*

	Feeding period	exp.	Number of species	
			Bacteria	Fungi
Feeding through the parafilm membrane	30 min.	1	2	0
		2	2	0
	1 hr.	1	2	0
		2	2	0
	10 hrs.	1	1	0
		2	2	0
	24 hrs.	1	1	0
		2	1	0

This experimental result shows that there is bacterial contamination of the feeding solution caused by the insertion of stylets.

II. Microorganisms on aphid body

An aphid which had fasted for 3 hours was placed in a Petri-dish (9 cm in diameter) containing the medium of Bouillon agar or PDA. After free walking on the medium for 10 minutes, the aphid was removed. Bacterial and fungal colonies on these agar medium were counted after incubation at 25°C for 2 days and 5 days, respectively.

The results are shown in Table 2. Six species of bacteria and 6 species of fungi were detected in this experiment.

TABLE 2. *Microorganisms isolated from aphid body*

exp.	Isolated colony type and numbers	
	Bacteria	Fungi
1	6	6
2	5	6
3	6	4

III. The number of bacteria in the feeding solution

The number of bacterial cells increasing in the feeding solutions was counted. Sterilized 2% sucrose solution or distilled water of 2 ml were put into sterilized parafilm membrane bag, and aphids were allowed to feed through the membrane. After free feeding for 30 minutes, 24 hours and 48 hours by 300 aphids at room temperature, the aphids were removed. The feeding solution of 0.2 ml was applied on a Bouillon agar medium and then spread on the surface of the agar with a sterilized glass stick. Incubation was carried out at 25°C, and the number of bacterial colonies was counted at 2 days after the inoculation.

The results are shown in Fig. 2. Many bacteria were found in the feeding solutions of a feeding period for 30 minuts or more. The sterilized 2% sucrose solution was better for bacterial growth compared with the sterilized distilled water. In the case of the sterilized 2% sucrose solution fed for 48 hours, about 600 bacterial cells were detected from 0.2 ml of feeding solution. Therefore, the number of bacterial cells in 100 ml was calculated to be about 300,000 cells. From the above mentioned results, it is obvious that the solution fed by the aphid for 2 days contains a large amount of bacteria.

IV. Inhibitory effect of the feeding solution on virus infection

Until now, the feeding solution obtained by the method cited above is condensed and has been used for experiments of the inhibitory action on the virus infection.

The aphid was allowed to feed on sterilized destilled water for 24 hours through

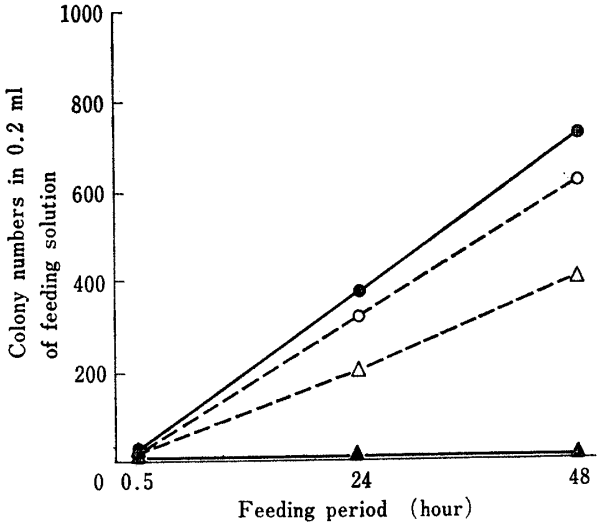

Fig. 2. The number of bacteria in feeding solution
•——• : Not feeding, unsterilized 2% sucrose solution
▲——▲ : Not feeding, sterilized 2% sucrose solution
o- - - -o : Feeding, sterilized 2% sucrose solution
△- - - -△ : Feeding, sterilized distilled water

the parafilm membrane. The collected solution of 250 ml was concentrated to 5 ml, and this concentrated solution was defined as the original solution and diluted with sterilized distilled water for the experiment. Ten grams of infected broad bean leaves were homogenized with 10 ml of 0.01 M phosphate buffer (pH 7.0) and squeezed with a gauze. And then the solution was centrifuged at 10,000×g for 20 minutes. This supernatant was used as the virus solution. Two ml of the supernatant was mixed with 2 ml of each diluted solution. Mechanical inoculation with carborundum was performed immediately on the leaf of *C. amaranticolor* by the half-leaf method. The virus solution diluted with sterilized water was rubbed on the other half side of the leaf as the control. Inhibitory effect on the infection was determined by the number of local lesions which occurred on the *C. amaranticolor* leaves. The rate of inhibition was defined as follows:

$$\left(1-\frac{\text{number of lesions in treatment}}{\text{number of lesions in control}}\right)\times 100\%$$

The results are shown in Fig. 3. Inhibition was found only in the case of the original solution diluted 2 times finally, and it indicated the inhibitory rate of about 20%. Other diluted original solutions did not show the inhibition.

V. Inhibitory effect by cultured medium of the bacterium

The inhibitory effect on virus infection by bacteria or bacterial products was investigated under various cultural conditions. In the succeeding experiments, the most predominant isolate in contaminated bacteria was used.

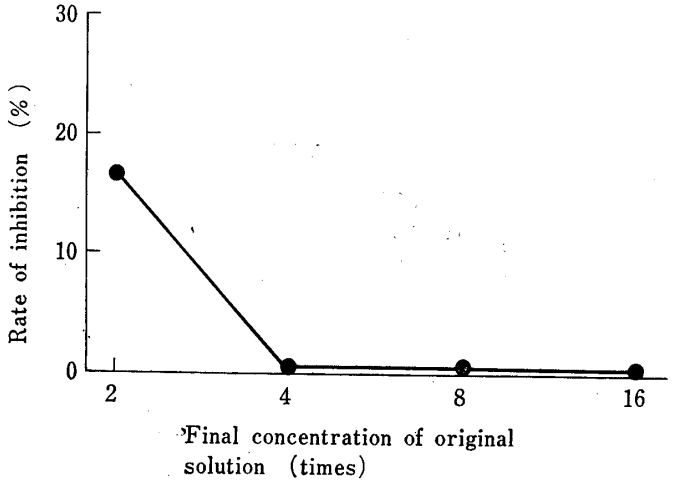

Fig. 3. Inhibitory effect of the feeding solution on virus infection

1) Effect of Husain and Kelman medium inoculated with the bacteria

The bacteria were inoculated in 500 ml of Husain and Kelman medium (pH 7.0), and incubated at 25°C for 10 days. After the incubation, cultured medium was filtrated through a filter paper (Toyo No. 2) and concentrated into 10 ml at 45°C by using a rotary evaporator (Tokyo Rikakikai Co., Ltd.). This concentrated solution was used as the original solution. Non-incubated Husain and Kelman medium was used for the control, and 500 ml of this medium was concentrated by the same treatment. The BYMV solution was the juice of infected leaves as indicated above. The original solution was diluted with a sterilized distilled water from 2 times to 32 times, and these diluted solutions of 5 ml and virus solution of 5 ml were mixed respectively. Using the half-leaf method, the mixture was inoculated on the leaf of *C. amaranticolor* and the solution of the same virus concentration diluted with a distilled water was prepared as the control. A similar procedure was followed also in the case of the non-inoculated medium. The inhibitory effect of virus infection was estimated by the number of the local lesions on the *C. amaranticolor* leaves. As the damage of the inoculated leaves seemed to be caused by the sucrose contained in the condensed cultural solution, injured leaves were checked in every experiments. Thus the effect of the sucrose solution containing the same concentration as each mixture of virus solution and cultured solution was investigated also.

These results are shown in Table 3 and 4. The mixture of the original solution and the virus solution caused a withering or a severe injury on inoculated leaves. Therefore, it was not possible to distinguish whether or not the necrotic lesions were true local lesions on these damaged leaves. The mixture which was diluted 4 times finally caused slight injury on 17 out of 20 leaves, but the appearance of local lesions was inhibited remarkably at the rate of 98.6%. No injury of the leaf was found in the solution diluted 16 times in final and this solution

TABLE 3. *Inhibitory effect by cultured solution of the bacterium*

Final concentration of diluted original solution	Final concentration of sucrose	Injured leaf numbers		Inhibition rate (%)	
		C	B	C	B
2 (times)	0.85 M	20/20*	20/20*	—	—
4	0.43 M	9/20	17/20	54.5	98.6
8	0.22 M	0/20	4/20	8.5	98.3
16	0.11 M	0/20	0/20	0	54.9
32	0.06 M	0/20	0/20	4.2	10.2
64	0.03 M	0/20	0/20	1.9	0

B: Inoculated cultural solution
C: Non-inoculated cultural solution
* Numerator: the number of leaves injured (or withered); denominator: number of treated leaves.

TABLE 4. *Injurious effect on leaf by sucrose*

Final concentration of sucrose	Injured leaf numbers	Withered leaf numbers
0.85 M	30/30*	30/30*
0.43 M	14/30	6/30
0.22 M	0/30	0/30
0.11 M	0/30	0/30
0.06 M	0/30	0/30
0.03 M	0/30	0/30

The legend refers to Table 3.

indicated an inhibitory rate of 54.9%. The inhibitory effect was scarecely observed in 32 times diluted solution and concentrated non-inoculated medium added the virus solution, and the original solution which was mixed with the virus oslution caused severe withering or injury to the inoculated leaves. The inhibitory rate of 54.5% was found in concentrated non-inoculated cultural solution diluted 4 times. However the inhibitory effect was scaresely found in the same solution diluted 8 times or more.

These results indicate that the solution from concentrated non-inoculated medium has a lower rate of inhibition than that of the concentrated bacteria-cultured medium. So that it was presumed that there were any inhibitory substances of virus infection in bacteria-cultured medium. For the observation of the effect of the sugar, the same concentration of sucrose as that of the medium were used. The solutions containing various sugar contents were rubbed with carborundum on the leaves of *C. amaranticolor* respectively. Withering was observed in all leaves rubbed with a 0.85 M sucrose solution. By rubbing with 0.43 M sucrose solution, 6 leaves were withered and 8 leaves were injured of the 30 leaves treated. But there were no effects on the rubbed leaves in the case of solutions which were less than 0.22 M of sucrose.

It seemed that the occurrence of withering and injury in inoculated leaves

observed in the experiments of concentrated bacteria-cultured medium and non-inoculated medium was due to the dense sucrose content in the solutions used for the inoculation.

2) Effect of distilled water inoculated with the bacteria

From the above cited experiments, it is clear that the bacteria were introduced into the feeding solution through the parafilm membrane, and that the feeding solution had an inhibitory effect on virus infection. However, as many nutrients were contained in Husain and Kelman medium except the sucrose, the inhibitory effect by these nutrients may have remained. For that reason, distilled water was used for the culture medium. The bacteria were cultured on the agar medium in a test tube for 10 days and the bacterial colonies in 5 test tubes were transferred into 200 ml of sterilized distilled water. After the incubation for 10 days at 25°C, the incubated water was centrifuged and the supernatant was concentrated into a volume of 10 ml under reduced pressure, and this concentrated solution was used as the original solution of super natant. The sediment was resuspended in 5 ml of sterilized distilled water, and this solution was used as the original solution of sediment. Each original solution was diluted from 2 times to 32 times with sterilized distilled water. Two ml of each diluted solution and 2 ml of partially purified virus solution were mixed respectively. These mixtures were rubbed on leaves of *C. amaranticolor* with carborundum using the half-leaf method.

The results are shown in Fig. 4. Concentrated supernatant inhibited the infection. The inhibitory rate was 30% with the solution diluted 2 times and 15% with the solution diluted 16 times. On the contrary, when the original suspension of the sediment was diluted 2 times, the inhibition of the infection was slight and the rate was 10%. From these results, it was presumed that the inhibi-

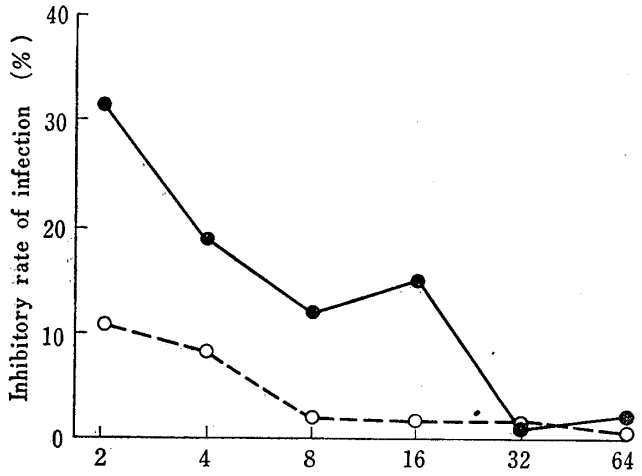

Fig. 4. Inhibitory effect for virus infection by concentrated bacteria-cultured distilled water

•——• : Supernatant ○- - - -○ : Sediment

tory effect to virus infection was caused by bacterial products or by substances by the autolysis in sterilized water.

3) Effect of sucrose solution inoculated with the bacteria

To remove the effect of sucrose from concentrated solution, the investigation was carried out by using the culture of the bacterium in sterilized 1% sucrose solution.

The bacterium was cultured in test tubes for 10 days and the bacterial colonies in 5 test tubes were transferred into 200 ml of sterilized 1% sucrose solution. After incubation at 25°C for 24 and 48 hours respectively, each cultured solution was centrifuged at 5,000 rpm for 10 minutes. Each supernatant was concentrated to 100 ml under reduced pressure, and in this case the sucrose concentration of the concentrated solution was 2%. These solutions were defined as the original solution of each supernatant. Each sediment was resuspended in 50 ml of sterilized 2% sucrose solution respectively, and the resuspensions were defined as the original solution of sediment. Each original solution was diluted with distilled water from 2 times to 32 times and the suspensions of partially purified virus were mixed with those diluted solutions of 5 ml in equal amount respectively. Using the half-leaf method, every mixed solution was inoculated on the leaf of *C. amaranticolor*.

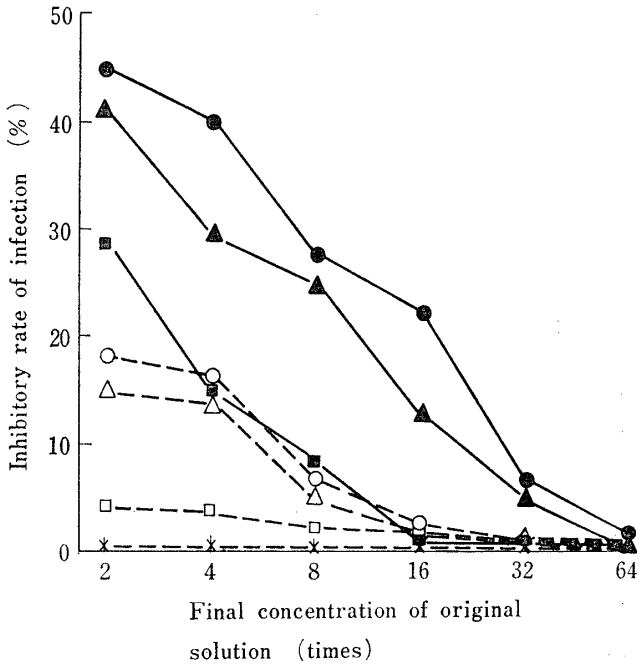

Fig. 5. Inhibitory effect for virus infection by concentrated bacteria-cultured sucrose solution

- ●——● : Supernatant-72 hr incubation
- ▲——▲ : Supernatant-48 hr incubation
- ■——■ : Supernatant-24 hr incubation
- ○- - -○ : Sediment-72 hr incubation
- △- - -△ : Sediment-48 hr incubation
- □- - -□ : Sediment-24 hr incubation
- ✱- - -✱ : Sterilized 2% sucrose solution

Sterilized 2% sucrose solution or sterilized distilled water mixed with virus suspension were used as the control. Local lesion numbers were counted and the inhibitory rate of the infection was calculated.

The results are shown in Fig. 5. The inhibitory effect of supernatants of every incubation period was greater than that of the suspensions of sediment. When the original solution of supernatant which had been incubated for 72 hours was diluted 2 times, this solution inhibited the infection at the rate of about 45%. A rate of about 20% was observed, when the solutions was diluted 16 times. And it was observed that the inhibitory rate of the supernatant increased with the incubation period. On the other hand, it seemed that slight inhibitory effect by sediment solution was derived from bacterial products containing in the sediment.

VI. Inactivation of the virus in solution by feeding

Whether the virus is inactivated directly by any substance in the cultured solution or indirectly by its effect to the epidermal cells was not obvious. To elucidate the former question, the following experiment was performed about the effect of inactivation of the virus in the solution by feeding of the aphid.

Partially purified virus solution of 2 ml was put into sterilized 12 parafilm membrane bags, and 6 bags were used for feeding and not feeding respectively. Three hundred aphids for a tube were allowed to feed through the parafilm membrane. After feeding for a given period, 0.2 ml of the feeding solution was spread on a Bouillon agar medium with a sterilized glass stick. Incubation was carried out at 25°C for 2 days, and the number of bacterial colonies were counted. The remaining solution of 1.8 ml was inoculated mechanically on the leaf of *C. amaranti-*

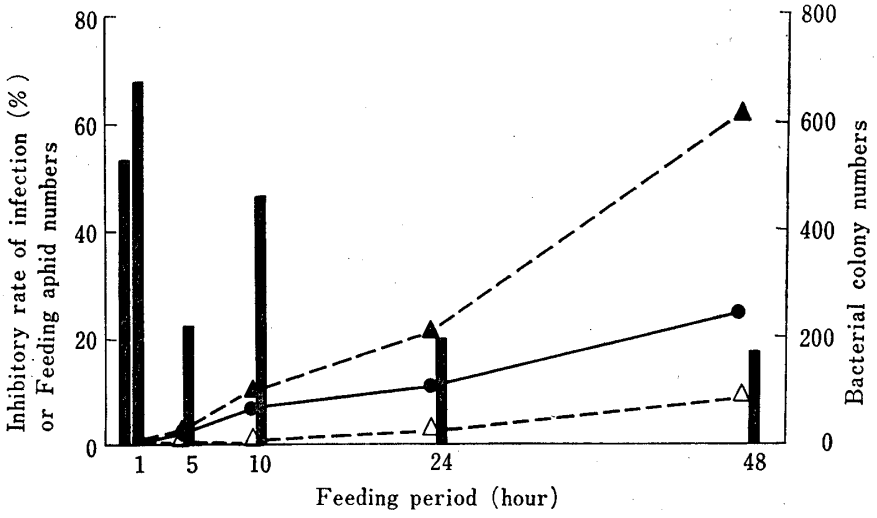

Fig. 6. Inactivation of the virus in solution by feeding
▲----▲ : Feeding, bacterial colony
△----△ : Not feeding, bacterial colony
●——● : Inhibitory rate of virus infection
▬ : Feeding aphid numbers

color by the half-leaf method, and not feeding virus solution was inoculated as the control. The number of feeding aphids was counted at each given time.

The results are shown in Fig. 6. The virus activity in the fed solution decreased gradually with the increasing of the feeding period. The activity decreased by 50% due to feeding for 48 hours. The inhibitory effect was not observed in the feeding period of 5 hours but observed over 10 hours. On the other hand, the number of bacteria in the fed solution increased rapidly after the feeding period of 24 hours, and about 6,000 colonies of bacteria were observed at 48 hours in the fed solution of 2 ml. In the virus solution without feeding, 2 species of bacteria were found. These bacteria were different in color and shape of colony from the bacteria contaminated by feeding. Therefore it is presumed that these bacteria had been contaminated in the partially purified virus pellet. As the number of aphids which fed through the membrane was few in general, the secreted saliva volume seemed to be very minute. So that the loss of virus activity by feeding was thought to be brought about by the bacterial products which increased rapidly rather than the action of the saliva secreted into the feeding solution.

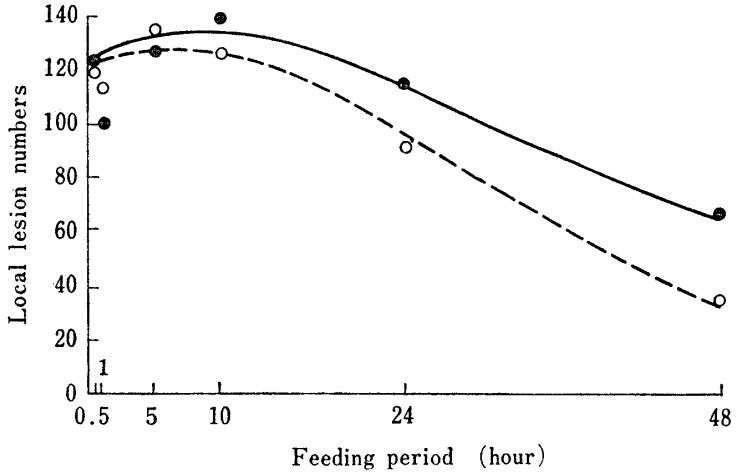

Fig. 7. Natural breakdown of virus activity
•——• : Partially purified virus solution
o- - -o : Partially purified virus solution fed by aphids

Fig. 7 shows the natural breakdown of virus activity at room temperature. Virus activity which was indicated by necrotic lesions decreased gradually after 10 hours. And at 48 hours, the virus inactivation rate of about 40% was observed. In the case of the virus solution fed by aphids, about 75% of the virus activity was lost at 48 hours compared with the initial activity.

The results indicates that inactivation of the virus was accelerated by feeding.

Discussion

It has been thought generally that the watery saliva brought some effects to the inserted plant cells or virus itself. But role of aphid saliva on the virus transmis-

sion has not been well known. For the purpose of elucidating this phenomenon, it is necessary to collect the watery saliva secreted by aphids, but there are no methods to collect the saliva itself more efficiently in a large amount. For this reason, the clarification of the relation of the saliva and virus infection is very difficult at present.

Nishi (1963) obtained the saliva by free feeding for three days through a rubber membrane. He used green peach aphids and 3% sucrose solution for feeding. After the feeding for 3 days, the solution was mixed with TMV and inoculated the mixture on *N. glutinosa* by half-leaf method. The inhibitory rate of virus infection was 52.7–52.9%. He explained that this was the effect of saliva introduced into sucrose solution. Nakazawa (1965) performed similar experiments using the saliva of *Myzus persicae* Sulz. and *Aphids gossypii* Glover obtained from feeding solution through a thin rubber or parafilm membrane. CMV infection was inhibited by the feeding solution and this inhibitory effect varied with combination of virus strains and aphid species.

The fact that the saliva had a cellulose-hydrolyzing effect was reported by Adams and Drew (1965) using the fed solution of aphid through a parafilm membrane. But they mentioned that it was obscure whether this enzyme was derived from salivary gland cells or from microorganisms introduced into the feeding solution.

In our laboratory, Hashiba and Misawa (1970) collected the saliva using the usual method through the parafilm membrane, and studied it's inhibitory effect on virus infection. However, doubt of microbial contamination in the feeding solution still remains. So that this questionable point was investigated in this paper.

It is properly considerable that the microorganisms adhere to the aphids body. The authors made this fact clear and presumed that these microorganisms vary by the habitat and species of aphids. And also the existence of microorganism on stylet surface became clear, and it was indicated that these microorganisms were introduced into the feeding solution by usual methods of saliva collection through the membrane. Our experimental results showed the contamination of bacteria in the feeding solution. Moreover bacterial cell numbers increased with increasing of feeding period. When the feeding solution was mixed with virus solution, the virus infection was inhibited. Therefore it is presumed that this inhibition is due to bacterial products in the feeding solution.

However, Moericke *et al.* (1965) performed the experiment using saliva in sucrose solution fed through the parafilm membrane with two species of aphids. From our experiments, the solution which they obtained seems to be unuseable as the pure salivary solution.

Virus activity was inhibited when aphids fed directly on a partially purified virus solution through the parafilm membrane. This fact is presumed attributable to the virus inhibiting effect by bacterial products mixed into virus solution.

It is not appropriate to use the solution which is obtained from concentrated or alcohol-sedimented aphid saliva obtained by usual method as a salivary component. From the above mentioned conclusions, we see that the method of obtaining aphid saliva needs further development.

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